Characterization of charge heterogeneity of antibody -drug conjugate by anion-exchange chromatofocusing

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□ ABSTRACT □

An anion-exchange chromatofocusing method was developed for the analysis of charge heterogeneity of antibody-drug conjugates. Different chromatographic conditions were evaluated including pre-gradient volume, column temperature, pH of start and elution buffers, and addition of pharmalytes 8-10.5. The charge isoforms were separated with acceptable resolution with the developed chromatofocusing method. The chromatofocusing profile of the studied conjugated antibody demonstrated its charge heterogeneity with p*I* values ranged from 7.3 to 8.5. The developed chromatofocusing method was applied for monitoring the stability of studied conjugated antibody under stress conditions.

Keywords: Antibody, Conjugate, Charge Isoforms, P*i*, Chromatofocusing.

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توصيف عدم تجانس الشحنات لجسم مضاد مرتبط مع دواء باستخدام الكروماتوغرافيا البؤرية مبادلة الأيونات السالبة

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□ ملخّص □

طورت طريقة كروماتوغرافيا بؤرية باستخدام أعمدة مبادلة للأيونات السالبة بهدف دراسة عدم تجانس الشحنة لجسم ضد مع دواء. قيم تأثير تغيير الشروط الكروماتوغرافية المختلفة بما في ذلك حجوم ما قبل الشطف المدروج، ودرجة حرارة العمود، وpH المحاليل الدارئة البادئة والمستخدمة للشطف، وإضافة محاليل اللأمفوليت ذات المجال 8–10.5. تم فصل النظائر المختلفة في الشحنة مع معامل فصل مقبول باستخدام طريقة الكروماتوغرافيا البؤرية المطورة. أظهر المرتسم الكروماتوغرافي البؤري للجسم المضاد المرتبط مع دواء المدروس وجود عدد من النظائر المختلفة في pI والتي تتراوح قيمها بين 7.3 و8.5. تم تطبيق طريقة الكروماتوغرافيا البؤرية المطورة لمراقبة ثبات الجسم المضاد المرتبط مع دواء المدروس الخاضع لشروط ثبات قاسية.

الكلمات المفتاحية: الجسم المضاد، المرتبط مع دواء، نظائر مختلفة الشحنات، pI، الكروماتوغرافيا البؤرية.

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Introduction

Antibody-drug conjugates (ADCs) are ideal candidates for is targeted prodrug therapy which is a promising approach to achieve a more selective treatment [1]. ADCs are monoclonal antibodies (mAbs) linked to cell-killing drugs. Thanks to their high binding specificity for tumor-specific antigens, mAbs can be used as vehicles to target cell-killing payloads to tumor cells [2-16]. Unique or overexpressed, tumor-specific antigens can be found in a wide range of human tumor cells. Some mAbs have the ability to recognize and specifically bind to these tumor-associated antigens. They can be used as single agents for the treatment of cancer through binding to cancer-cell-specific antigens and induction of an immunological response against the target cancer cell [15-16]. However, therapeutic efficacy is often limited by the extent to which the antibody leads to cell death. Monoclonal antibodies are extremely discriminating for their targets but sometimes therapeutically ineffective on their own. The insufficient efficiency of most naked mAbs in cancer therapy has been circumvented by arming the immunoglobulin with radioactive isotopes or cytotoxic drugs, yielding highly specific ADCs.

One of the challenges in production of therapeutic ADCs is to demonstrate product consistency after process changes such as cell line, production scale, conjugation reaction, manufacturing site, formulation, purification process, etc [17]. When these changes are made either during development or post-commercialization, a comparability study is required to ensure product consistency and safety for patients.

ADCs display considerable heterogeneity resulted from various modifications in a protein structure of antibody itself, such as deamidation, amino acid substitution/deletion, differential glycosylation, glycation, etc. Furthermore, the conjugation process by linking several drug molecules to antibody increases the heterogeneity of ADC. Such modifications may result in the presence of many different species in crude ADC preparations and in final, purified products. Therefore, the ADCs should be characterized for charge heterogeneity, size heterogeneity, and for peptide mapping as possible using relevant methods [19].

One of the molecular characteristics of ADCs is the charge heterogeneity. Several analytical methods could be used to characterize the charge heterogeneity of ADC including ion exchange chromatography (IEC) [20-22], Isoelectric focusing (IEF) [23], and capillary electrophoresis (CE) [24].

The aim of this study was to determine the p*I* and charge distribution profile of a novel humanized antibody conjugated to a cytotoxic maytansine derivative. To achieve this objective, a chromatofocusing method using anion exchange column was developed to characterize the charge profile of conjugated antibody.

Materials and Methods

Reagents: the used reagents were Diethanolamine (Sigma-Aldrich), Hydrochloric acid (VWR), Polybuffer 96 (GE healthCare), NaCl (Acros).

Protein samples: a monooclonal antibody naked and conjugated to the maytansinoid were analysed.

Chromatofocusing apparatus

A Dionex ICS-3000 chromatographic instrument controlled by Chromeleon® software (version 6.80) was used for all analyses. All the instrument components were obtained from Dionex (Sunnyvale, CA, USA). Other experimental conditions were: column: Mono P 5/200 GL (particle size: 10 µm, 200*5mm I. D) (GE Healthcare)., flow rate 1ml/min, λ:

280nm, and injection quantity: 200 or 500µg. The applied gradient used in all the study was presented in Table 1.

Table 1 presents the gradient used in all the study.

Steps	Time (min)	Flow rate (ml/min)	%A	%B	%C
Elution	0	1	0	100	0
	25,00	1	0	100	0
Wash	25,50	1	0	0	100
	33,50	1	0	0	100
Equilibration	34,00	1	100	0	0
	61,50	1	100	0	0

As there is no pI markers in this method, pH of the fractions collected was measured in order to estimate the pI of charge isoforms.

Results ad discussion:

Analysis of charge variants is used to characterize ADC. Monitoring the charge homogeneity of ADC provide information on protein stability and purity from batch to batch, the pathways of degradation, etc. The charge heterogeneity of studied ADC could be resulted from various modifications in the protein structure of antibody itself, and from the conjugation process. In this work, we focused on the characterization of charge distribution profile of studied ADC by chromatofocusing.

Chromatofocusing methods mainly based on using ion exchange stationary phases (essentially anion exchangers). This method permits to separate proteins in order of their isoelectric point (pI) by generation a pH gradient through the separation medium [25-28].

In the case of anion exchanger chromatofocusing, the column is equilibrated with a start buffer, usually alkaline. Then, an elution buffer (acidic) travels through the column and generates a decreasing pH gradient. In most cases, the elution buffer contains a mixture of polymeric buffering species for board and narrow pH intervals. With decreasing pH mobile phase, proteins will elute from the column when the pH reaches their pI where they have little to no charge. A further decrease in pH during the gradient will make the protein more cationic and therefore repletion occur between the protein and the column resin.

In preliminary experiments, the mobile phase was composed of: (A): Start buffer (diethanolamine-hydrochloric acid diluted in deionised water 0.025M, pH 9.5), (B): Elution buffer (Polybuffer 96-hydrochloric acid, diluted in deionised water 10%, pH 6.0) and (C): washing phase (NaCl 2M). The optimization of chromatofocusing separation of charge isoforms of conjugated monoclonal antibody was performed by modifying some conditions: pre-gradient, column temperature, pH of start and elution buffers, and addition of pharmalytes 8-10.5. The results of modification of different parameters were presented in table 2.

Table 2. Obtained results of optimised parameters of the chromatofocusing method for the separation of studied conjugated antibody isoforms.

Parameter	Optimization	Results	Retained conditions
Pre-gradient	0, 5, 10 min	no improvement of studied ADC isoforms separation	Work Without pre-gradient
Column temperature	20, 25, 30°C	no improvement of studied ADC isoforms separation	Work at 20°C
Start buffer pH	9.5, 10, 10.5	Slight improvement of studied ADC isoform separation by increasing start buffer pH from 9.5 to 10.5	10.5
Elution buffer pH	6, 7	No improvement of studied ADC isoform separation by using more narrow pH rang (10.5-7) instead of 10.5-6	6
Addition of Pharmalyte 8-10.5 in different %	0% 0.2% 0.4% 0.8%	Improvement of studied ADC isoform separation by using 0.2% Decrease of resolution of isoform separation by increasing % Pharmalyte 8-10.5 from 0.2% to 0.8%	0.2%

The results of different chromatographic conditions mentioned in table2 showed that the best conditions of the mobile phase composition were: (A): Start buffer (0.025M diethanolamine-hydrochloric acid diluted in deionised water, pH 10.5), (B): Elution buffer (Polybuffer 96-hydrochloric acid, diluted in deionised water (8%), Pharmalyte 8-10.5 (0.2%), pH 6.0) and (C): washing phase (2M NaCl). Figure 1 presented the obtained chromatogram of naked AB and its ACD in the best conditions mentioned above.

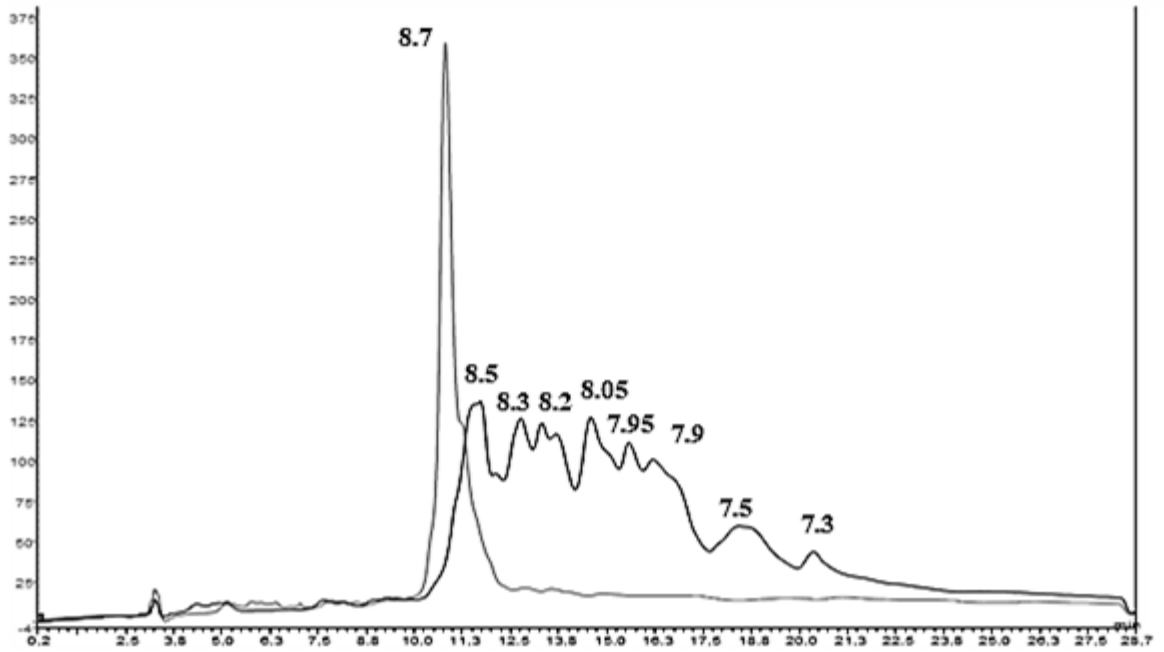

Figure 1. Chromatofocusing profile of naked AB and ADC. Experimental Conditions: Column: Mono P 5/200 GL, (particle size: 10 µm, 200* 5mm I. D) (GE Healthcare). Phase mobile: Phase A, 0.025M Diethanolamine, pH 10.5, HCl. Phase B: Pharmalyte 8-10.5, 0.2%, Polybuffer 96 (8%) pH 6, HCl. Phase C: 2M NaCl. Column temperature: 20°C, λ: 280 nm. Injected quantity: 200µg (naked AB), 500µg (ADC). Injector temperature: 5 °C. Flow rate : 1mL/min.

In the optimized conditions, the comparison between the chromatofocunsing profiles of naked AB and its ADC demonstrated that the ADC was more heterogeneous and acidic than corresponding naked antibody. This behaviour is related to the fact that the covalently linking of cytotoxic drug to the free amine groups of lysine of these mAbs decreases their pI each time a lysine residue is modified by a linker molecule. Furthermore, this conjugation process increases the charge heterogeneity of resulted ADCs.

To establish the degradation pathways and the intrinsic stability of the studied ADC, stress testing was carried out one month in pH 5.5 (room temperature and 40°C), and pH 8 (room temperature). The t0 samples have been frozen at -80°C. The optimized conditions of chromatofocusing were applied to these stressed samples and the results were presented in figure 2.

The chromatofocusing profiles of ADC under stress testing showed that ADC in pH 5.5 was stable at -80°C (t0) and at room temperature but it was not stable in pH 8 at -80°C (t0). An increase of percent of basic species of ADC was remarked at pH 8 although the ADC was stored at -80°C. ADC in pH 5.5 was not stable one month at 40°C. There was an increase of percent of acidic species and a decrease in percent of basic species ADC as compared to t0 (Figure 2).

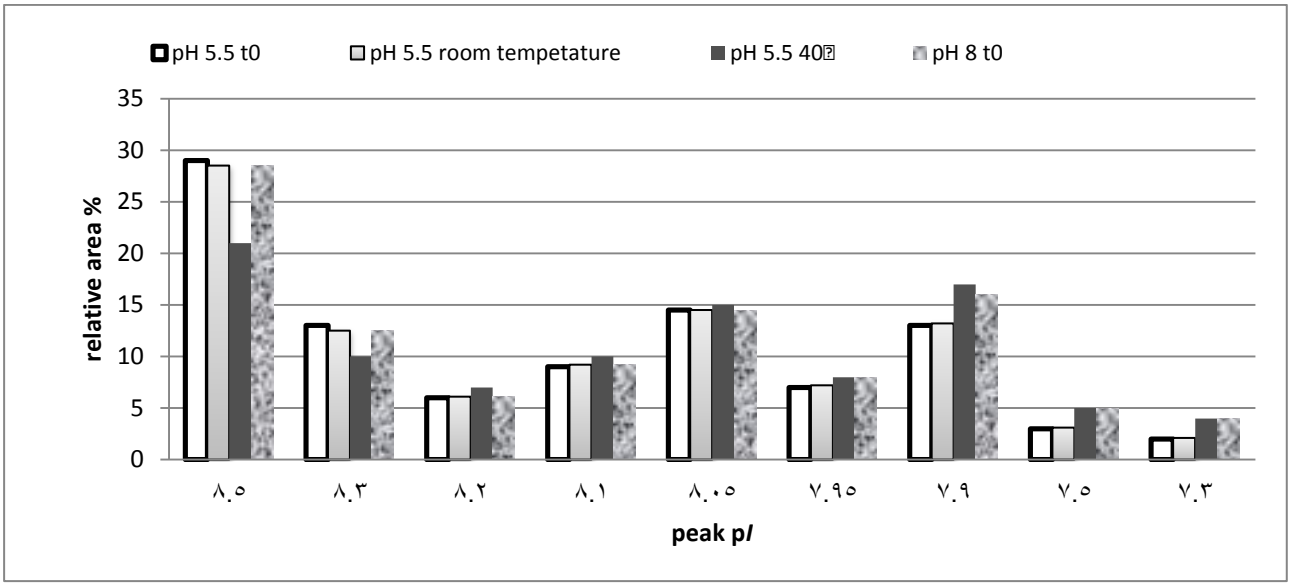

Figure 2. Comparison of % isoform of ADC in pH 8 -80°C (t0) and in pH 5.5 at -80°C (t0), room temperatureand 40°C. Other conditions as mentioned in figure 1.

Conclusion

An anion chromatofocusing method was developed for the characterization of charge distribution profile of ADC. The developed chromatofocusing method revealed that studied ADC has several charge species of pI values ranged from 7.3 to 8.5. These charge isoforms were separated with good resolution using the optimized conditions. The developed method were applied to evaluate the stability of studied ADC under stress conditions.

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